PB90-108267

Intact Soil-Core Microcosms for Evaluating the Fate and Ecological Impact of the Release of Genetically Engineered Microorganisms

Idaho Univ., Moscow

Prepared for:

Corvallis Environmental Research Lab., OR

1989

U.S. Department of Commerce
National Technical Information Service
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ERL-COR- 881J

TECHNICAL REPORT DATA
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1. REPORT NO. EPA/600/J-89/105	2.
3. PB90-108267	
4. TITLE AND SUBTITLE: Intact soil-core microcosms for evaluating the fate and ecological impact of the release of genetically engineered microorganisms	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): S.A. Bentjen,[1] J.K. Fredrickson,[2] P. Van Voris,[2] and S.W. Li[2]	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: [1]Univ. of ID, Moscow, and [2]Pacific Northwest Laboratory, Richland, WA	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO. IAG DW89931854
12. SPONSORING AGENCY NAME AND ADDRESS: US Environmental Protection Agency, Environmental Research Laboratory, 200 SW 35th Street, Corvallis, OR 97333	13. TYPE OF REPORT AND PERIOD COVERED: Journal Article
	14. SPONSORING AGENCY CODE: EPA/600/2

15. SUPPLEMENTARY NOTES

Applied and Environ. Microbiology 55(1): 198-202

16. ABSTRACT

Intact soil-core microcosms were studied to determine their applicability for evaluating the transport, survival, and potential ecosystem effects of genetically engineered microorganisms before they are released into the environment. Soil-core microcosms were planted with wheat and maize seeds and inoculated with *Azospirillum lipoferum* SpBr17 and SpRG20a Tn5 mutants, respectively. Microcosm leachate, rhizosphere soil, plant endorhizosphere, insects, and xylem exudate were sampled for *A. lipoferum* Tn5 mutant populations. *A. lipoferum* Tn5 populations, determined by most-probable-number technique-DNA hybridization, varied from below detection to 10^5 g of dry root^{-1} in the rhizosphere, with smaller populations detected in the endorhizosphere. Intact soil-core microcosms were found to maintain some of the complexities of the natural ecosystem and should be particularly useful for initial evaluations of the fate of plant-associated genetically engineered bacteria.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL INFORMATION SERVICE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT: Release to Public	19. SECURITY CLASS (This Report): Unclassified	21. NO. OF PAGES: 6
	20. SECURITY CLASS (This page): Unclassified	22. PRICE

EPA Form 2220-1 (9-73)

EPA/600/J-89/105

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 198–202
0099-2240/89/010198-05$02.00/0
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Vol. 55, No. 1

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Intact Soil-Core Microcosms for Evaluating the Fate and Ecological Impact of the Release of Genetically Engineered Microorganisms

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Received 2 May 1988/Accepted 18 October 1988

Intact soil-core microcosms were studied to determine their applicability for evaluating the transport, survival, and potential ecosystem effects of genetically engineered microorganisms before they are released into the environment. Soil-core microcosms were planted with wheat and maize seeds and inoculated with *Azospirillum lipoferum* SpBr17 and SpRG20a Tn*5* mutants, respectively. Microcosm leachate, rhizosphere soil, plant endorhizosphere, insects, and xylem exudate were sampled for *A. lipoferum* Tn*5* mutant populations. *A. lipoferum* Tn*5* populations, determined by most-probable-number technique-DNA hybridization, varied from below detection to 10^6 g of dry root^{-1} in the rhizosphere, with smaller populations detected in the endorhizosphere. Intact soil-core microcosms were found to maintain some of the complexities of the natural ecosystem and should be particularly useful for initial evaluations of the fate of plant-associated genetically engineered bacteria.

Methods are needed to evaluate genetically engineered microorganisms (GEMs) in representative ecosystems to assess their fate and potential effects before they are released into the environment. Laboratory-contained microcosms have potential as risk assessment tools prior to field testing. Current philosophy in regard to the release of GEMs into the environment is that each organism should be regulated on the basis of the risk posed by that individual organism, rather than by how that organism was altered (17). A standard test system must be adaptable to evaluate the release of a wide variety of microorganisms as well as detect possible detrimental effects on the ecosystem. Microcosms serve these purposes and can also be used to determine the efficacy of GEMs without the regulatory constraints of field testing. Intact soil-core microcosms have been used to study ecosystem stability (29), nutrient cycling (28), and the effect of utility wastes or other toxic substances on ecological processes (7, 26a, 27). Microcosms have also facilitated the study of microorganism-pollutant interaction in aquatic (20) and sediment ecosystems (9). Whereas chemical substances are dispersed, diluted, or degraded following their introduction into the ecosystem, microorganisms can reproduce and increase in population under favorable conditions. Therefore, microorganisms have the potential to spread and colonize habitats that may be unique to each genotype.

The transposon Tn*5* was used to mutagenize the *A. lipoferum* strains to enhance their detection and enumeration in the microcosms. The presence of Tn*5* in the bacterial genome confers kanamycin resistance (11), which can be used as a selective marker and also as a unique DNA sequence to positively identify mutants by DNA hybridization with a Tn*5*-specific probe (5). In addition, transposon mutants were chosen for these studies because they have been widely used to produce mutants for studying bacterial symbiosis with plants (22), for plant-pathogen interactions (3, 4, 13), and for determining bacterial survival in soil (25). This study was initiated to determine the applicability of intact soil-core microcosms for evaluating GEM transport through the ecosystem, colonization of the rhizosphere and endorhizosphere, displacement of other indigenous rhizosphere microorganisms, and effects on plant nutrient uptake.

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MATERIALS AND METHODS

Bacterial cultures, media, and maintenance. *Escherichia coli* MV12, containing the suicide plasmid pGS9 (23), was used as the Tn*5* donor in matings. *A. lipoferum* SpRG20a and SpBr17, ATCC 29708 and 29709, respectively, were purchased from the American Type Culture Collection, Rockville, Md. Luria-Bertani (LB) broth (15), containing 25 μg of kanamycin sulfate ml^{-1} (Sigma Chemical Co., St. Louis, Mo.) and of chloramphenicol ml^{-1} (Sigma) was used for maintenance of *E. coli* MV12. *Azospirillum* cultures were maintained on *Azospirillum* medium (AM), which consisted of nitrogen-free medium (R. Cote, ed., ATCC media handbook, 1st ed., p. 42. American Type Culture Collection, 1984) supplemented with 1.0 g of NH_4Cl and 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.) liter^{-1}. *Azospirillum* Tn*5* mutants were routinely cultured on AM supplemented with 25 μg of kanamycin sulfate ml^{-1}. Stock cultures of all bacteria were maintained at −80°C in 50% glycerol.

Transposon mutagenesis of *A. lipoferum* strains and physical characterization of mutants was conducted by the methods described by Vanstockem et al. (26). Repeated transfer of the different *A. lipoferum* Tn*5* mutants on N-free AM indicated that the mutants were able to fix N_2 and grow at the same rate in pure culture as the wild type.

Microcosms and inoculation. Intact soil cores for use as microcosms were obtained as described by Van Voris (26a), with a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Tex.). The intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on Büchner funnels (24-cm-diameter polypropylene funnel [Dynalab Corp., Rochester, N.Y.] or 20-cm-diameter porcelain funnel [Coors, Golden, Colo.]). Two soil-plant combinations common to agricultural regions of eastern Washington were used. Cores (17.8 by 60 cm) of Palouse silt loam soil (fine-silty, mixed, mesic Pachic Ultic Haploxeroll) from the Palouse region were planted with spring wheat (*Triticum aestivum*), while cores (25 by 60

cm) of Burbank loamy fine sand soil (sandy, skeletal, mixed, xeric Torriorthent) common to the Columbia Basin were planted with maize (*Zea mays*). Common local varieties, NC+3390 maize (Wilbur-Ellis Seed Co., Pasco, Wash.), and Edwall spring wheat (U.S. Department of Agriculture Research Station, Pullman, Wash.) were used.

Tn*5* mutant cultures were washed in 0.85% sterile saline and suspended in sterile deionized water to obtain a density of 9×10^8 and 4×10^8 CFU ml^{-1} for *A. lipoferum* SpBr17 or SpRG20a, respectively. Burbank soil microcosms were planted with four maize seeds per microcosm, with each seed receiving 1.0 ml of an equal mixture of three *A. lipoferum* SpBr17 Tn*5* mutants (17-1, 17-3, and 17-6). Palouse microcosms were planted with five seeds per microcosm, with each seed receiving 1.0 ml of a single *A. lipoferum* SpRG20a Tn*5* mutant. Heat-killed suspensions of the same cells served as controls. There were six replicates for each treatment in a completely randomized design, and one-way analyses of variance tables were calculated to determine if statistically significant differences ($P \leq 0.05$) existed between measured parameters in inoculated versus control microcosms. Microcosms were maintained at a water potential of approximately −0.03 MPa and a temperature of 22°C, as monitored by soil temperature and moisture probes on line to a datalogger (model 21X [Campbell Scientific Co., Logan, Utah]). The photoperiod was set for 12 h, with an initial light intensity at the soil surface of 400 microeinsteins m^{-2} (4100 K Ultralume lamps [Phillips Lighting Corp., Bloomfield, N.J.]).

Insects. Insects were introduced onto plants after the first sampling. Ten European corn borers (*Ostrinia nubilalis*) (French Agricultural Research Service, Lamberton, Minn.), at the third to fourth instar, were placed on individual maize plants and were contained to the lower stalk by loosely wrapping with chiffon fabric. Approximately 50 to 100 aphids (*Schizaphis* sp.) indigenous to eastern Washington were introduced onto wheat plants. After 12 and 21 days for corn borers and aphids, respectively, insects were sampled, macerated in sterile deionized water, and assayed for *A. lipoferum* Tn*5* mutants. Approximately 25 aphids were removed from the wheat leaf surface, and from 4 to 9 corn borers were removed from the maize stem interior.

Sampling frequency and methods. Three replicate microcosms from each treatment were sacrificed at 4 and 9 weeks to determine the fate of *A. lipoferum* Tn*5* mutants. Soil cores were removed from the pipe intact, and root samples with adhering soil were taken randomly throughout the core with care to avoid sampling roots growing at the interface between the core and the pipe. Standard methods were used for sampling rhizosphere soil (31). Rhizosphere populations of *Rhizobium leguminosarum* biovar *viciae* were enumerated by the most-probable-number plant infectivity method of Weaver and Frederick (30) with peas (*Pisum sativum*) as the host plant. Nitrifying bacteria in the rhizosphere were enumerated by the most-probable-number microtechnique of Rowe et al. (21). Endorhizosphere analysis for Tn*5* mutants (19) was accomplished by placing washed wheat and maize root sections in 1.0% chloramine T (Sigma) for 15 and 60 min, respectively, followed by a 30-min wash in sterile 0.1 M (pH 7.0) phosphate buffer. Roots were then rinsed three times with sterile deionized water, rolled on AM agar plates with 150 μg of kanamycin ml^{-1} and 200 μg of cycloheximide (Sigma) ml^{-1} to check for efficiency of surface disinfestation of the *A. lipoferum* mutants, macerated, and evaluated for the presence of *A. lipoferum* Tn*5* mutants.

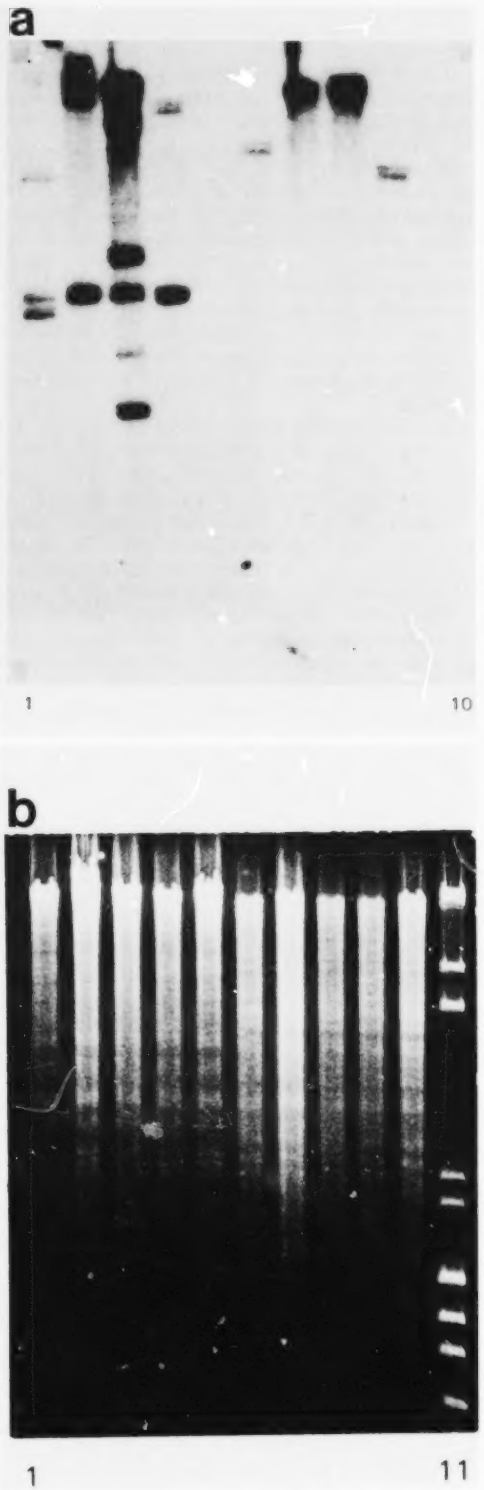
Xylem exudate was collected after cutting plants 5 cm from the base of the stem with sterile scissors, letting fluid collect on the stem surface for several minutes, and then removing it with a sterile pipette tip and spread plating immediately. Leachate was collected in sterile, acid-washed, deionized water-rinsed, 2-liter Erlenmeyer flasks after saturating the cores with deionized water. The procedure was to place microcosm leachate at 4°C within 12 h of breakthrough and entry into the flasks. Leaf tissue sampling for N analysis was conducted by standard methods (10). Leaf N concentration was determined by Kjeldahl analysis (2) prior to maize tasseling and wheat booting.

Enumeration of *Azospirillum* Tn*5* mutants. Rhizosphere, endorhizosphere, xylem exudate, insects, and leachate samples were analyzed for the presence and population of *Azospirillum* Tn*5* mutants. Colony blots (6), as well as most-probable-number (MPN) technique-DNA hybridization (5) were used for enumeration. All blots were hybridized with [α-^{32}P]dCTP-labeled probe and autoradiographed for enumeration. As whole pGS9 probe proved nonspecific, a 30-base-pair sequence in the *nptII* region of Tn*5* was used as a probe. The *nptII* probe was synthesized (model 380A [Applied Biosystems, Foster City, Calif.]) and purified by polyacrylamide gel electrophoresis (15% polyacrylamide and 7 M urea) by the protocol of the manufacturer. Kinase end labeling (15) with [γ-^{32}P]ATP (Dupont, NEN Research Products, Boston, Mass.) routinely achieved 10^8 dpm μg of DNA^{-1}.

RESULTS AND DISCUSSION

Transposon insertions have been shown to impair the relative competitiveness of *Rhizobium leguminosarum* (F. J. Brockman, M.S. thesis, Washington State University, Pullman, Wash., 1988) and therefore, Tn*5* insertion could have had a negative impact on the competitive ability of the *A. lipoferum* mutants used in this study. Alternatively, Tn*5* mutant *E. coli* has been shown to outcompete the wild type in the absence of any known selective advantage (1). To reduce the likelihood of introducing an impaired organism, a mixture of three different mutants was used for inoculating the Palouse soil-core microcosms. In order to select these mutants, the physical location of Tn*5* within the mutant genome was determined. Whole chromosomal *A. lipoferum* Tn*5* mutant DNA restriction digests (Fig. 1) with *Eco*RI and *Hin*dIII, which give 0 and 2 cuts in Tn*5* (11), respectively, demonstrated that each mutant used in the inoculum mix had a single copy of Tn*5* inserted uniquely in the genome (Fig. 1). The one exception was mutant 17-6, which possessed pGS9 vector sequences, as demonstrated by the increase in the total number of *Hin*dIII-generated fragments hybridizing to pGS9, three of which correspond to the same size fragments generated by *Hin*dIII-generated fragments of pGS9.

MPN technique-DNA hybridization was, in general, more effective than the combination of plate counts and colony blotting for enumerating *A. lipoferum* Tn*5* bacteria when high background levels of Km^r bacteria were present. In some samples, hybridization was observed for low dilutions from microtiter plate blots while no colonies probed positive on colony blots of dilution plates of the same samples. Presumably, the static broth provided *Azospirillum* cultures more favorable conditions for growth and competition with other Km^r bacteria. *Azospirillum* cultures seemed to be especially overgrown by the indigenous Km^r bacteria on solid medium. Typically, the indigenous Km^r bacteria would appear after 2 days of incubation at 27°C, while a minimum of 3 days was required for *Azospirillum* colonies. Addition-



ally, not all colonies blotted efficiently, requiring their purification and isolation before hybridization with the Tn*5* probe for confirmation.

Leachate from microcosms receiving heat-killed cells contained bacteria other than *A. lipoferum* that grew on AM supplemented with 150 μg of kanamycin ml^{-1} and 200 μg of cycloheximide ml^{-1}, some of which had homology with pGS9. Studies with *Pseudomonas putida* Tn*5* mutants added to dried and remoistened Palouse soil enumerated by MPN technique-DNA hybridization did not reveal homology of indigenous bacteria with whole pGS9 probe (5). In that study, either homologous bacteria were not able to grow on the medium used for *P. putida* Tn*5* selection, or the bacteria were not present because of air-drying of the soil. Specificity in conjunction with appropriate controls is essential for sensitive enumeration of specific bacterial populations in environmental samples using DNA probes.

To determine whether the homology was due to Tn*5* sequences in indigenous soil bacteria or to vector sequences, a 30-base-pair sequence in the *nptII* region of Tn*5* was used as a probe. Isolated bacteria homologous to the probe when spot blotted and hybridized with pCU101 (pGS9 without the Tn*5* DNA sequence) gave a strong hybridization signal, while a duplicate blot did not hybridize with the 30-bp *nptII* probe, indicating that homology was with vector sequences (data not shown). Thus, the *nptII* probe was used for detection and enumeration of *Azospirillum* Tn*5* mutants.

Rhizosphere populations of the *Azospirillum* Tn*5* mutants in the inoculated microcosms were variable, ranging from undetectable to 10^6 g^{-1} (dry weight) (Table 1). Endorhizosphere populations as determined by MPN technique-DNA blots at midpoint sampling were below the detection limit for wheat roots and ranged from 5.5×10^3 to 6.5×10^3 CFU g of dry maize $root^{-1}$ in the Burbank soil microcosms. Chloramine T-treated root samples that were rolled on AM containing 150 μg of kanamycin ml^{-1} and 200 μg of cycloheximide ml^{-1} prior to maceration showed no growth, indicating that no viable *Azospirillum* Tn*5* mutants were on the root surface. Although *Azospirillum* rhizosphere populations were not uniform between replicate microcosms, a pattern was evident. Of the three replicate Palouse and Burbank microcosms receiving live inoculum at each sampling, two microcosms had detectable rhizosphere populations of *A. lipoferum* Tn*5*, while in one microcosm, populations were below the level of detection.

Variability of *Azospirillum* Tn*5* rhizosphere populations between replicates was likely due to inherent difference in the microcosms. Intact soil cores, because they are removed as discrete pieces from the field, contain inherent variation representative of the complexity of that ecosystem. A few examples of macroscopic differences that were observed in some microcosms and not others include visible layers of Mount St. Helens ash from the 1980 eruption, the emergence of mushrooms, and the presence of earthworms. Microscopic differences could include variable distribution of

FIG. 1. Agarose gel electrophoresis of chromosomal restriction digests of *A. lipoferum* Tn*5* mutants (a) and autoradiogram of vacuum-transferred DNA hybridized with a pGS9 DNA probe (b). Lanes 1 through 5 contain *Hin*dIII restriction digests of Tn*5* mutants 17-1 (lane 1), 17-3 (lane 2), 17-6 (lane 3), 20a-2 (lane 4), and *A. lipoferum* SpBr17 wild type (lane 5). Lanes 6 through 10 contain *Eco*RI restriction digests of Tn*5* mutants 17-1 (lane 6), 17-3 (lane 7), 17-6 (lane 8), 20a-2 (lane 9), and *A. lipoferum* SpBr17 wild type (lane 10). Lane 11 contains ϕX174 *Hae*III and lambda *Hin*dIII molecular weight markers.

TABLE 1. Populations of *A. lipoferum* Tn*5* mutants in the rhizosphere of maize and wheat at 33 and 67 days after seeding

Treatment, time, and replicate no.	*A. lipoferum* rhizosphere population (log MPN g of dry soil^{-1})
Maize on Burbank soil	
33 days	
1	4.55
2	3.79
3	BD[a]
67 days	
4	3.78
5	BD
6	4.23
Wheat on Palouse soil	
33 days	
1	6.43
2	BD
3	BD
67 days	
4	4.40
5	3.75
6	BD

[a] BD, Below detection.

indigenous soil bacteria (e.g., antibiosis) and distribution of nutrients.

No *Azospirillum* Tn*5* mutants were detected in leachate, xylem exudate, insects, or earthworms found fortuitously during microcosm sampling. Xylem exudate and insects had low background populations of Km^r bacteria, as did Burbank microcosm leachate samples; hence, the Tn*5* mutants, if present and culturable, would have been readily detected by colony hybridization or MPN technique-DNA hybridization. Populations determined by plate count and MPN technique-DNA hybridization were generally in agreement, although the latter gave more consistent and slightly higher (typically 50%) values. Indigenous bacteria not hybridizing with Tn*5* DNA and able to grow on AM supplemented with antibiotics ranged from 0 to 10^6 g of soil^{-1} in the Palouse rhizosphere; hence, the relatively slow-growing *A. lipoferum* population may have been outcompeted, limiting its detection.

There was no effect ($P \leq 0.05$) on plant leaf nitrogen concentration as a result of inoculation with *Azospirillum* Tn*5* mutants. Plant growth effects from *Azospirillum* inoculation have been attributed to N_2 fixation (16), production of plant growth hormones (24), and increased nutrient uptake at the roots (16). The expression of these traits and limited root colonization likely contribute the variability in plant growth response common to *Azospirillum* studies. In addition, the degree of root colonization by *Azospirillum* Tn*5* mutants can be dependent on the strain used (18). Likewise, plant genotype-*Azospirillum* interactions have been shown to be strain specific for maize (8).

The potential for niche displacement of rhizosphere populations of nitrifying bacteria and *R. leguminosarum* in the microcosms was also evaluated because of their involvement in biochemical N cycling and the ability of *R. leguminosarum* to be stimulated in the rhizosphere of non-host plants. There were no significant differences between control and inoculated treatments in *Rhizobium* populations (10^2 to 10^4 MPN g of dry soil^{-1}) or nitrifying bacteria (10^4 to 10^5 MPN g of dry soil^{-1}) in the rhizosphere. Although changes in the rhizosphere populations of nitrifying bacteria and *R. leguminosarum* were not observed in the inoculated microcosms, their consistent presence and low variability between replicates make them potential indicators of ecosystem stability.

The *A. lipoferum* strains were chosen for their reported xylem mobility (12, 14); hence, there was a potential for transmission from the rhizosphere through the plant vascular system and to phytophagous insects. In the specific *Azospirillum*-soil-plant combination used in this study, extensive bacterial movement and colonization were not observed. Although the possibility exists that mutations incurred by the insertion of Tn*5* influenced the competitive ability of the bacteria, and therefore xylem mobility, it is more likely that the migration of *Azospirillum* mutants into the xylem is uncommon and occurs only under specific conditions. Levels of the *A. lipoferum* Tn*5* mutants below detection in xylem exudate and in phytophagous insects suggest that this would not be a significant mode of *Azospirillum* transport through the ecosystem.

We have shown in this study that intact soil-core microcosms maintain many of the features useful for evaluating GEM transmission through the ecosystem and colonization of the rhizosphere and endorhizosphere, carriage by insect vectors, potential displacement of other rhizosphere microorganisms, and effects on plant growth. One illustration of this is the hybridization of whole pGS9 to indigenous Km^r bacteria, when no such problem was observed in other studies using dried stock soil (5). This phenomenon is indicative of the higher order of ecosystem complexity maintained by use of intact soil-core microcosms (29). Other aspects of environmental release not evaluated in this study that may be evaluated by using soil-core microcosms include GEM persistence and gene transfer. A major advantage of microcosms for GEM evaluation is their easy replication and the ability to maintain appropriate controls, necessary to determine the probability of finding a given effect or population for risk assessment. The use of intact soil cores allows the testing and comparison of GEM fate and effects in a number of different soil types, including those at the site intended for field testing.

ACKNOWLEDGMENTS

This work was supported by the U.S. Environmental Protection Agency-Corvallis Environmental Research Laboratory under a Related Services Agreement with the U.S. Department of Energy under contract DE-AC06-76RLO 1830.

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